AD_____

Award Number: DAMD17-97-1-7050

TITLE: Cloning of Novel Oncogenes Involved in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Channing Der, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
at Chapel Hill
Chapel Hill, North Carolina 27599-1350

REPORT DATE: June 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2000	Annual (1 Jun 99 - 30 May 00)

4. TITLE AND SUBTITLE
Cloning of Novel Oncogenes Involved in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7050

6. AUTHOR(S)
Channing Der, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-1350

E-MAIL:
cjder@med.unc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

During the past year, we initiated our own efforts to utilize cDNA expression libraries in retrovirus vectors to screen for novel oncogenes. We have collected 8 breast cancer samples, representing either localized, noninvasive tumor tissue from invasive cancers. The rationale for choosing the noninvasive samples was that this screen would involve the identification of oncogenes involved in tumor progression, and hence, may serve as important diagnostic markers for early detection. The rationale for using the invasive tumor tissue is that these cancer cells are likely to harbor more genetic defects that noninvasive tumor cells and consequently, our chances of detection are increased. A second rationale for studying late stage cancers is that oncogenes involved promoting invasion and metastases may be identified. The identification of such genes are obviously important as both diagnostic markers for metastases and as targets for drug discovery.

14. SUBJECT TERMS
Breast Cancer, CDA Award

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

6/27/02

PI - Signature Date

Table of Contents

Introduction

Cancer is a multi-step genetic process involving mutation of oncogenes and tumor suppressor genes. With a few notable exceptions the genetic determinants which contribute to the development of breast cancer remain unknown. Conventional techniques for identifying oncogenes in human tumor cells are cumbersome, inefficient and have met with limited success. Moreover, for practical reasons they have relied on the transformation of fibroblast cell lines to identify oncogenes in epithelial cell-derived tumors. Thus, it seems likely that many oncogenes present in epithelial cell-derived breast carcinomas may not produce an obviously transformed phenotype in fibroblast cells. These limitations argue that oncogenes and tumor suppressor genes that contribute to transformation in human breast cancer remain to be detected. The development of retrovirus vector-based cDNA libraries overcomes the key obstacles to expression cloning of oncogenes contributing to the transformation of epithelial cells. Thus, we propose to generate retroviral cDNA libraries from human breast tumor cells and screen them for DNA sequences which contribute to the transformation of breast epithelial cells. We believe that this approach will lead to the successful identification of new genetic markers for breast cancer and identify novel targets for the rational design of anti-cancer drugs against breast cancer.

Body

This grant was originally a career development award that funded a former Research Assistant Professor in our research group (Dr. Geoffrey J. Clark) that was then transferred to the current PI this past year. Consequently, we have modified some of the approaches that were outlined in his original proposal for our studies. During the past year, we initiated our own efforts to utilize cDNA expression libraries in retrovirus vectors to screen for novel oncogenes. We have collected 8 breast cancer samples, representing either localized, noninvasive tumor tissue or tumor tissue from invasive cancers. The rationale for choosing the noninvasive samples was that this screen would involve the identification of oncogenes involved in tumor progression, and hence, may serve as important diagnostic markers for early detection. The rationale for using the invasive tumor tissue is that these cancer cells are likely to harbor more genetic defects than noninvasive tumor cells and consequently, our chances of detection are increased. A second rationale for studying late stage cancers is that oncogenes involved in promoting invasion and metastases may be identified. The identification of such genes are obviously important as both diagnostic markers for metastases and as targets for drug discovery.

A key change from the original proposal is the use of patient-derived breast cancer tissue for generation of the cDNA expression libraries. Progress has involved the isolation of 8 different breast cancer tissue samples for RNA isolation. These represent both noninvasive and invasive tumor tissue. Four cDNA libraries have been prepared and two have been introduced into the pCTV3 retrovirus vector. One has been characterized for the frequency and size of inserted cDNA (Fig. 1). Once verified that the library contained suitable insert cDNA sequences, it was used to infect NIH 3T3 mouse fibroblasts for the detection of focus-formation.

Progress on Task A, to generate and screen cDNA retrovirus libraries will continue into the next year. However, during the course of our studies, we encountered technical difficulties regarding the isolation of cDNA sequences from transformed cells. We have also embarked on further development of a human breast epithelial cell system for our biological assays. As

described below, while multiple transformed foci were identified in our screens with NIH 3T3 cells, we have put the analyses of these on hold while we develop this potentially more relevant screen for oncogenes.

A. Biological screens for novel oncogenes

An important strength of our use of retrovirus vectors is the opportunity to utilize a broader range of cell types and biological assays for detection of genes that promote various facets of oncogenesis. Our initial screens were done with NIH 3T3 rodent fibroblasts. Advantages of using these cells are their sensitivity to transformation by a wide variety of functionally diverse oncoproteins and their ease of infection by retroviruses. However, a potentially significant limitation of these cells is their tendency towards spontaneous transformation. In our first two screens for oncogenes using NIH 3T3 cells, with one breast tumor library, we did identify over 30 different transformed foci. However, spontaneously arising foci were also seen with the empty retrovirus control cultures. We have isolated, using cloning cylinders, over 30 foci of transformed cells (Fig. 2). These cultures clearly retain a transformed growth phenotype after subcloning, a characteristic that makes it unlikely that they represent spontaneously-arising background foci. We have expanded these cell isolates and they have been stored in liquid nitrogen.

Another potential limitation of using NIH 3T3 cells for these screens is that fibroblasts may not be susceptible to transformation by genes important for carcinoma development. Therefore, work during the past year has involved the development of epithelial cell lines for our screens. We initially analyzed two mouse mammary epithelial cell lines for oncogene sensitivity. We used C127 and NMuMG mouse cell lines and evaluated their sensitivity to a variety of oncogenes. However, each cell line suffered from a significant spontaneous transformation background problem. Therefore, we have decided not to pursue the use of these cell lines for our screens.

We have found that another non-mammary epithelial cell line, the RIE-1 rat intestinal epithelial cell line, lacks any spontaneous transforming activity, and hence appears to give us a solid screen for our libraries. We determined that RIE-1 cells can be transformed in one hit by activated tyrosine kinases (e.g., Src) or GTPases (Ras), but not serine/threonine kinases (e.g., Raf). When we used RIE-1 cells in a separate screening of cDNA libraries generated from a patient-derived AML or a human head and neck carcinoma cell line, we isolated 8 different transformed foci from the AML library and 4 distinct transformed foci with the head and neck library. No background foci were seen on parallel cultures infected with the empty retrovirus. Therefore, while a mammary-derived epithelial cell line would be more appropriate for our screens, we feel that the use of any epithelial cell type would be advantageous over using fibroblasts.

We are also assessing and developing human MCF-10A breast epithelial cells for our screening. However, we decided to avoid the use of amphotropic viruses for our screening analyses using these cells. Therefore, one aspect of our ongoing work has been to generate MCF-10A human breast epithelial cells that harbor and express exogenously introduced ecotropic receptor. This will allow us to use ecotropic viruses for our screens. This is advantageous for two key reasons. First, the ecotropic packaging cell lines result in the production of infectious virus at higher titers. Higher titers allow us to screen a smaller number of cells. Second, ecotropic viruses can infect only rodent cells, hence, there will be minimal

biohazard concerns with these library analyses. To date, our MCF-10A cell lines infected with a retrovirus encoding the ecotropic receptor has not greatly enhanced the infection rate with ecotropic virus. The logical explanation for this is the lack of high expression of the receptor. Thus, the next step in this process will be to isolate individual subclones and assay each for infection efficiency.

B. Isolation of transforming cDNA sequences

The rate-limiting step, and the most technically demanding, in our screens has been the isolation of the transforming cDNAs from the transformed cells. During the past year, we have experienced some technical difficulties at two steps in this process. First, we have had difficulty in using PCR-mediated amplification to isolate the cDNA sequences. Second, in some cases where the cDNAs were isolated successfully, no transforming activity was detected in subsequent transformation assays. We discuss what we have done to try to overcome these limitations.

From the transformed cell populations isolated from the screen using NIH 3T3 cells, we have failed to efficiently isolate the transforming cDNAs from a majority of the isolates. One possible concern has been the quality of the genomic DNA that was isolated from the cells for PCR-mediated amplification using DNA primers corresponding to the flanking vector sequences. Therefore,we have tried a variety of different methods to isolate the genomic DNA. To date, this approach has not effectively overcome this problem. Another possible concern is with the DNA primers. Therefore, we have tried to alter the sequences of the primers to address this concern. However, when we tried a mock situation, where NIH 3T3 cells were transformed with a retrovirus harboring an activated H-Ras cDNA sequence, these primers did effectively result in the isolation of the cDNA. Thus, for now, we have placed the isolates from the NIH 3T3 screens aside until we resolve the problem.

The second limitation we have seen is that when the cDNA were isolated, a large percentage of them failed to show transforming activity when reintroduced into cells. We believe that this problem is associated with the likelihood that multiple retroviruses are infecting each cell. Therefore, in addition to the transforming cDNA, other nontransforming cDNAs are also likely to be isolated by PCR amplification. Thus, we need to go back to these cells and isolate different sized fragments from the PCR reaction in hopes that they might represent the correct transforming cDNA. However, we should emphasize that this effort is only worthwhile if we feel that the rate of false positives in our screens is very low. Otherwise, the other explanation for the lack of transforming activity associated with our isolates is simply that these are spontaneously transformed cells, independent of any introduced cDNA. As described above, background is certainly a problem with NIH 3T3 cells; that is why we have shifted our analyses to RIE-1 cells.

Key Accomplishments

- Isolation of 8 human breast cancer samples from both noninvasive and invasive cancers
- Generation of cDNA from RNA isolated from 4 tumor tissues sufficient for generation of retrovirus libraries of sufficient complexity
- Verification that one library representing cDNA from an invasive ductal carcinoma contained cDNA sequences of sufficient quantity and quality
- Isolation of over 30 transformed foci of cells in NIH 3T3 cells screened with one breast cDNA library
- Evaluation of three mouse epithelial cell lines for feasibility as recipients for library screening

Reportable Outcomes

Abstract presentation at the DOD Era of Hope Meeting, Atlanta, GA, June, 2000

Conclusions

We will complete the generation of all 8 cDNA retrovirus libraries, continue the library screening on NIH 3T3 cells, continue to develop MCF-10A cells as a recipient for library infections, and begin the cloning of the transforming sequences detected in the initial NIH 3T3 focus-formation assays.

Appendices

Figure1. Enzyme Digestion of Random Clones to Identify Percent of Inserts.

Figure 2. Foci from NIH 3T3 Cells Transduced with a Retroviral Breast Cancer cDNA Expression Library.

Fig. 1. Enzyme Digestion of Random Clones to Identify Percent of Inserts. Six of 6 clones from the library made from the 1 kB insert fragments, whereas 5 of 6 from the library made from 2 kB cDNA fragments contained an insert. Vector fragment is indicated by the arrows.

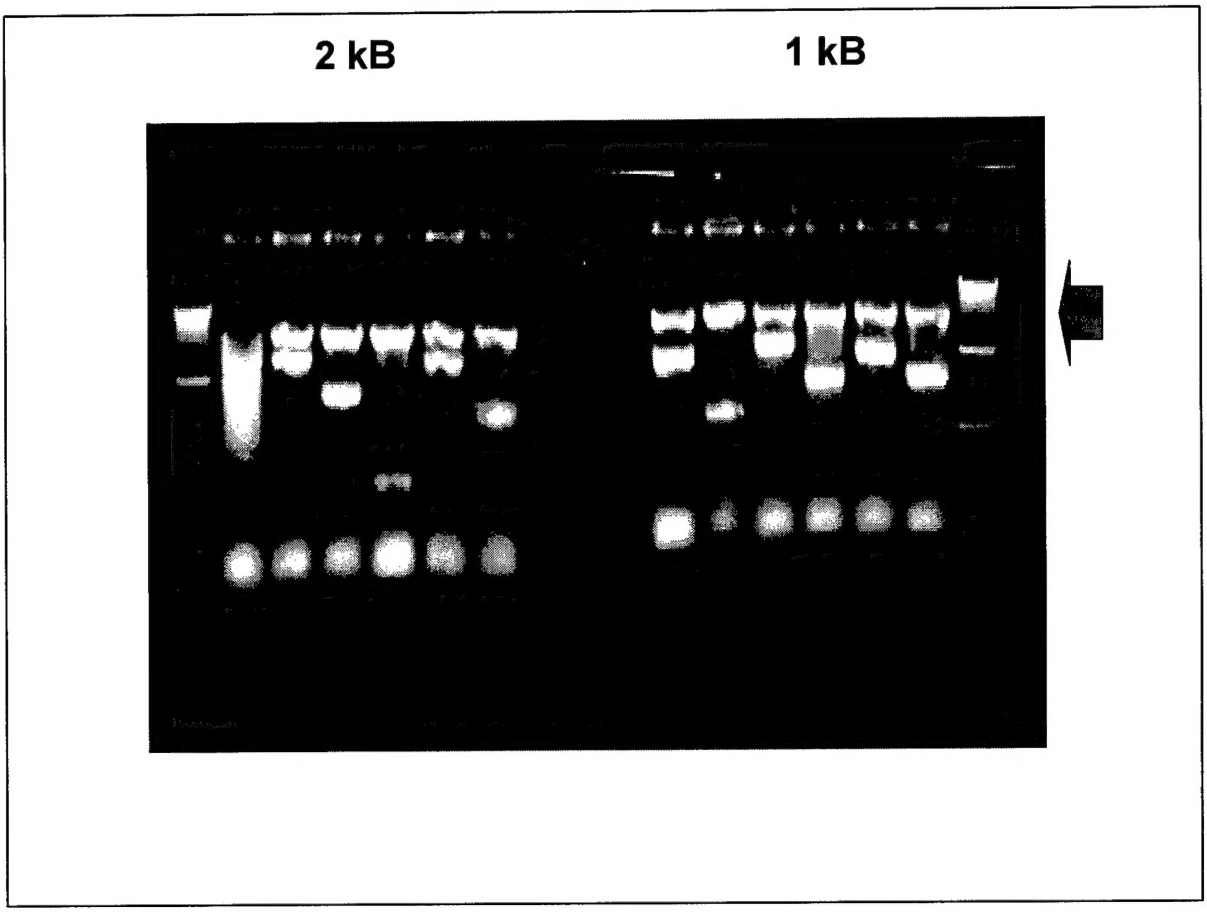

Fig. 2. Foci from NIH 3T3 Cells Transduced with a Retroviral Breast Cancer cDNA Expression Library. NIH 3T3 cells were infected with high titer virus prepared from BOSC cells transiently transfected with the plasmid DNA representing the retrovirus cDNA expression library. The appearance of foci of transformed cells was monitored for 14-18 days. Shown are 4 independent foci induced. The distinct appearance of these foci suggests that their transforming genes will exhibit distinct functions.